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Fluoride at Mitogenic Concentrations Increases the Steady State Phosphotyrosyl Phosphorylation Level of Cellular Proteins in Human Bone Cells*

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ABSTRACT

This study was designed to test the hypothesis that treatment of human bone cells with mitogenic concentrations of fluoride would lead to an increase in the steady state level of tyrosyl phosphorylation of specific cellular proteins. With an immunoblot assay method, it was found that mitogenic concentrations of fluoride (*i.e.* 50–200 μmol/L) induced a dose- and time-dependent increase in the level of tyrosyl phosphorylation of at least 13 cellular proteins in both normal human bone cells and human TE85 osteosarcoma cells. Time-course studies revealed that a statistically significant increase in tyrosyl phosphorylation of these 13 cellular proteins in human bone cells was observed after 3–6 h of fluoride treatment and was sustained for up to 24 h. This time course was not compatible with a direct activation of tyrosyl kinases, as epidermal growth factor, which activates tyrosyl kinase activity, induced an immediate and acute response that was rapidly reversible within 1 h. Although fluoride increased the steady state tyrosyl phosphorylation of the cellular proteins in human bone cells, the same micromolar doses of fluoride had no effect on human skin fibroblasts, which are fluoride-nonresponsive cells. The effects of fluoride were rapidly reversible in the absence of fluoride and could be acutely potentiated by pretreatment with epidermal growth factor. In summary, we have shown for the first time that mitogenic concentrations (*i.e.* 50–200 μmol/L) of fluoride increased the steady state level of tyrosyl phosphorylation of at least 13 cellular proteins in human bone cells, and that the increases were relatively slow in onset and sustained. In conclusion, these findings are consistent with the hypothesis that the osteogenic actions of fluoride are mediated at least in part by an inhibition of the activity of one or more fluoride-sensitive phosphotyrosyl protein phosphatases in human bone cells. (*J Clin Endocrinol Metab* **81:** 2570–2578, 1996)

FLUORIDE IS AN effective agent to increase spinal bone density in patients with osteoporosis (1). Past histomorphometric studies indicate that the effects of fluoride to increase bone formation were mediated by the stimulation of osteoblast proliferation (2). Previous biochemical studies from our laboratory (3, 4) and others (5–8) have demonstrated that fluoride at micromolar concentrations [similar to the effective serum fluoride concentrations in patients (9)] could act directly on osteoblasts of various species, including humans, to stimulate their proliferation *in vitro*. However, the molecular mechanism by which fluoride stimulates osteoblast proliferation has not been determined.

We have previously shown that an osteoblastic nonlysosomal acid phosphatase (ACP) was inhibited by mitogenic (micromolar) concentrations of fluoride (10, 11), and that this enzyme functioned as a phosphotyrosyl protein phosphatase (PTPP) at neutral pH (11–13). Based on the recent evidence that tyrosyl protein phosphorylation is closely associated with the cell proliferation process (14), and based on the concept that inhibition of tyrosyl protein dephosphorylation would in effect also increase the overall tyrosyl protein phosphorylation level, we have previously proposed a model for the biochemical mechanism of the mitogenic action of fluoride on bone cells (10, 15). In this model, we envision that the binding of a growth factor, *e.g.* insulin-like growth factor I, to the extracellular domain of its receptor activates the intrinsic tyrosyl kinase activity in the intracellular domain of the receptor. The activation of tyrosyl kinase activity would lead to increased tyrosyl phosphorylation of specific cellular proteins, which presumably mediates the mitogenic signal pathway. The increased tyrosyl phosphorylation of the mitogenic signaling proteins eventually results in an increase in cell proliferation. Under normal conditions, the osteoblastic fluoride-sensitive ACP/PTPP would be activated to dephosphorylate the mitogenic signaling tyrosyl proteins and thereby terminate the mitogenic signal, such that the increase in cell proliferation would dissipate. In the case of fluoride treatment, we postulate that fluoride would enter the bone cells and inhibit the activity of this fluoride-sensitive ACP/PTPP. Consequently, the mitogenic signaling tyrosyl proteins would not be dephosphorylated, the mitogenic signal would continue, and the increase in bone cell proliferation would be maintained.

Although the existing supporting evidence for the proposed model is strong, it is mostly circumstantial. In this regard, direct evidence that fluoride increases cellular tyrosyl protein phosphorylation levels is lacking. Accordingly, in this study we investigated the effects of mitogenic concentrations of fluoride on the steady state tyrosyl phosphorylation level of cellular

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proteins in human bone cells *in vitro*. The steady state level of cellular phosphotyrosyl proteins was measured by an immunoblot assay using a commercial antiphosphotyrosine antibody. In this study, we have shown for the first time that mitogenic concentrations (*i.e.* 50–200 μmol/L) of fluoride, under conditions that stimulated human osteoblastic cell proliferation, increased steady state phosphorylation level of at least 13 cellular phosphotyrosyl proteins in human bone cells in a dose- and time-dependent manner.

Materials and Methods

Materials

Tissue culture supplies were obtained from Falcon (Oxnard, CA), DMEM, bovine calf serum, penicillin, streptomycin, amphotericin B, as antibiotic-antimycotic, and trypsin were obtained from Life Technologies (Grand Island, NY). BSA (fraction V, RIA grade) was purchased from U.S. Biochemical Corp. (Cleveland, OH). Epidermal growth factor (EGF) was purchased from R&D Systems (Minneapolis, MN). [^{3}H]Thymidine (48 Ci/mmol) was obtained from Research Products International (Mount Prospect, IL). [^{125}I]NaI (2125 Ci/mmol), ammonium persulfate, sodium orthovanadate, and bromophenol blue were products of ICN Biochemicals (Costa Mesa, CA). Polyclonal antiphosphotyrosine antibodies were products of Promega Corp. (Madison, WI). Glycine, β-mercaptoethanol, tri(hydroxymethyl)aminomethane (Tris), SDS, Folin-Ciocalteu's phenol reagent, *N,N,N',N'*-tetramethyl-ethylenediamine (TEMED), and polyoxyethylene sorbitan monolaurate (Tween-20) were products of Sigma Chemical Co. (St. Louis, MO). Sodium fluoride was purchased from Spectrum Chemical Corp. (Gardena, CA). The enhanced chemiluminescence (ECL) detection kit was obtained from Amersham Corp. (Arlington Heights, IL). Fuji x-ray films were purchased through local suppliers. Immobilon-P transfer membrane was obtained from Millipore Corp. (Bedford, MA). Prestained electrophoresis standards were obtained from Bio-Rad Laboratories (Hercules, CA). All other reagents were of reagent grade and were obtained from Fisher Chemicals Co. (Los Angeles, CA).

Cell cultures

Normal human bone cells were prepared from human bone chip samples of mandible by collagenase treatment according to the method of Wergedal *et al.* (16). Human TE85 osteosarcoma cells, originally obtained from Dr. J. Fogh of Sloan-Kettering Institute (New York, NY), were maintained in our laboratory. Human skin fibroblasts were isolated from a human foreskin sample according to the method of Wergedal *et al.* (16).

Cell DNA synthesis assay

Human bone cells were plated at 2500 cells/cm^2 in 24-well plates in DMEM containing 10% bovine calf serum for 24 h. After plating, the bone cells were changed to serum-free DMEM and incubated for 24 h. After that, effectors (*e.g.* fluoride) were added, and incubation was continued for 24 h. Bone cell mitogenic activity was assayed by stimulation of incorporation of [^{3}H]thymidine (1.5 μCi/mL) during the final 2 h of the incubation. The assay was adapted from the method of Gospodarowicz *et al.* (17) and has been previously described (18). In some experiments, cell proliferation was confirmed by counting cell number using a hemocytometer after a 48-h incubation. The relative cell number was determined by counting the cells in 5 representative areas in each of the fluoride- and vehicle-treated cultured wells, and the average and SEM of six replicate cultures were reported.

Determination of steady state phosphotyrosyl protein level

Human bone cells were plated in 100-cm culture dishes in DMEM containing 10% bovine calf serum until cells reached approximately 70% confluence (*i.e.* approximately 10,000 cells/cm^2). The cells were then changed to serum-free DMEM and incubated for 16–24 h. After that, the cells were treated with various concentrations of fluoride for the indicated time period. After the treatment, cell medium was removed, and the cell layer was immediately extracted with 0.3–0.5 mL SDS treatment buffer [0.125 mol/L Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 1 mmol/L sodium orthovanadate, and 0.01% bromophenol blue]. The cell extracts were immediately placed in a boiling water bath for 2 min and kept frozen at −20 C until they were electrophoresed. Protein content in the extracts was determined according to the method of Lowry *et al.* (19).

Equal amounts (15–20 μg) of cellular proteins in each sample were separated on a 10% SDS polyacrylamide minigel. After electrophoresis, cellular proteins were transblotted onto an Immobilon-P membrane at 4 C at 40 V overnight. The nonspecific binding sites of the blotted membrane were blocked by incubation with 5% dry milk for 1 h. The blocked membrane was incubated with 0.5 μg/mL polyclonal antiphosphotyrosine antibody in 10% dry milk for 1 h at room temperature with constant shaking. [The antibody appeared to be specific for the phosphotyrosine moiety, as binding of the antibody to the phosphorylated EGF receptor in the human A431 epidermoid carcinoma cell membranes was completely blocked by coincubation with 5 mmol/L L-phosphotyrosine, but not by 5 mmol/L L-phosphoserine or 5 mmol/L L-phosphothreonine (data not shown).] The membrane was immediately rinsed twice with the TBS buffer (20 mmol/L Tris-HCl, pH 7.6, and 137 mmol/L NaCl), for 15 min each time, at room temperature with constant shaking. The membrane was incubated with the second antibody (horseradish peroxidase-linked antirabbit IgG), which was diluted 1:1000 in 5% dried milk at room temperature for 1 h. The blot was then rinsed five times with the blocking buffer (5% dried milk in TBS) for 15 min each time with constant shaking. The phosphotyrosine protein bands were visualized with the ECL detection assay kit. The steady state tyrosyl phosphorylation level was determined by measuring the relative intensity of the band compared to that of the corresponding band in the control using a 1D/2D Soft Laser Scanning Densitometer and ERIS Reporting Integrating System from Biomed Instruments (Fullerton, CA). The results were presented as a percentage of the corresponding control value.

For the EGF experiment, phosphotyrosine proteins bands were identified with ^{125}I-labeled protein A (radioiodinated by the chloramine-T method) (20). Briefly, the transblotted membrane, after incubation with the primary antibody, was rinsed once with the blocking buffer. ^{125}I-Labeled protein A (10^6 cpm/mL) was added to the blot and incubated for 1 h at room temperature. The blot was rinsed five times with the blocking buffer (15 min each time). Phosphotyrosyl proteins on the blot were identified by autoradiography with double Dupont Cronex Lightning Plus intensifier screens (DuPont, Wilmington, DE). The film was exposed for 24 h at −70 C. The relative intensity of the band was measured with the scanning laser densitometer.

To evaluate the sensitivity of the immunoblot assay to monitor small changes, we determined the intraassay variation and the confidence level of the assay. In this regard, an immunoblot was prepared from nine replicate human TE85 osteosarcoma cell layer extracts (*i.e.* from cells that had been cultured in DMEM containing 10% bovine calf serum). Nine major phosphotyrosyl proteins with apparent molecular sizes ranging from 21–220 kDa were selected for quantitation by scanning laser densitometry. As each cell extract had been treated separately, this procedure yielded nine replicate assessments for each of the nine phosphotyrosyl proteins. The average percent coefficient of variance for the cellular phosphotyrosyl proteins was 25 ± 6% (range, 16–35%), with no dependence on either the apparent molecular size of the cellular proteins ($r = -0.04$) or the relative band density ($r = -0.24$). On the basis of these results, we defined the 95% confidence limits for the means of duplicate values as a minimum difference of 48.5% (*i.e.* 1.96 × the maximum variation of 35%, divided by the square root of the sample size, $n = 2$). Accordingly, an observed difference in steady state tyrosyl phosphorylation level of greater than 48.5% in duplicate lanes may be considered significant at $P < 0.05$.

Statistical analysis

The statistical significance of the differences was determined by Student's two-tailed *t* test and ANOVA.

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Results

Stimulation of human bone cell proliferation by micromolar fluoride in vitro

Figure 1 demonstrates that fluoride at micromolar (50–200 μmol/L) concentrations significantly stimulated [^{3}H]thymidine incorporation into the DNA of human mandible-derived bone cells (A) and human TE85 osteosarcoma cells (B) in a biphasic manner, with maximal stimulation seen at 100 μmol/L fluoride. The increase in [^{3}H]thymidine incorporation was presumed to reflect a stimulation of cell proliferation, because treatment of human TE85 osteosarcoma cells with fluoride also increased cell number in a dose-dependent manner after a 48-h incubation (C).

Dose-dependent effects of fluoride on the steady state phosphorylation level of cellular phosphotyrosyl proteins in human bone cells in vitro

Figure 2A shows the steady state level of cellular phosphotyrosyl proteins in human TE85 cells after treatment with 0, 50, 100, or 500 μmol/L fluoride, respectively, for 24 h. The steady state tyrosyl phosphorylation level of at least 13 cellular proteins (with apparent molecular masses of 21, 32, 34, 42, 48, 62, 74, 105, 120, 150, 170, 220, and 240 kDa, respectively) was significantly increased by mitogenic doses (*i.e.* 50–200 μmol/L) of fluoride. The average densitometric measurements (mean ± SEM, from three independent experiments) of the relative levels of these 13 cellular proteins in TE85 cells are depicted in Fig. 2B. The effect of fluoride was dose dependent with the optimal dose of approximately 100 μmol/L. In addition, 24 h treatment with 100 μmol/L fluoride also significantly increased steady state tyrosyl phosphorylation level of 13 cellular proteins in the normal, untransformed, human mandible-derived bone cells with

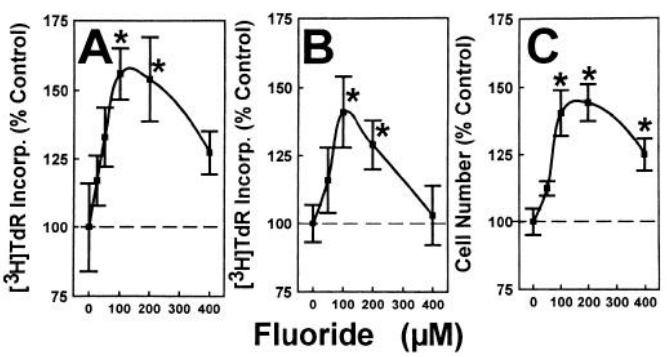

FIG. 1. Stimulation of human bone cell proliferation by fluoride. Human bone cell proliferation was measured either by increases in [^{3}H]thymidine incorporation after a 24-h incubation with fluoride (A and B) or by cell counting after a 48-h incubation with fluoride (C). A, Effects of the indicated concentrations of fluoride on normal human mandible-derived bone cells (mean ± SEM; n = 12). B, Effect of fluoride on human TE85 osteosarcoma cells (mean ± SEM; n = 6). C, Effect of a 48-h treatment with fluoride on the cell number of human TE85 cells (mean ± SEM; n = 6). The *dashed lines* represent 100% of the corresponding control values (in A, 100% control = 620 cpm; in B, 100% control = 527 cpm). In each case, 1% bovine calf serum increased [^{3}H]thymidine incorporation and cell number by 200–400% over the corresponding vehicle-treated control values. The statistical significance of the difference between the means was analyzed by Student's two-tailed *t* test. *, $P < 0.05$.

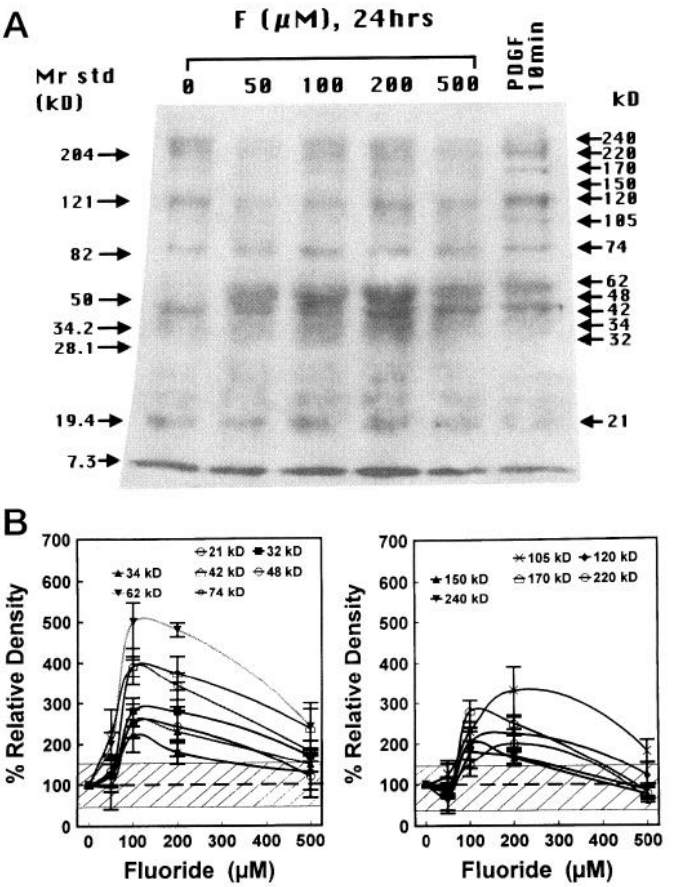

FIG. 2. Dose-dependent effects of fluoride on the steady state tyrosyl phosphorylation level of cellular proteins in human TE85 osteosarcoma cells. Human TE85 osteosarcoma cells were treated with the indicated concentrations of fluoride for 24 h. Treatment of the same cells with PDGF (10 ng/mL) for 10 min was included as a positive control. The steady state tyrosyl phosphorylation level was determined using the ECL detection method. A, Effects of fluoride treatment on the steady state tyrosyl phosphorylation levels of cellular proteins (indicated by the *arrows on the right*). The molecular mass standards are shown by the *arrows on the left*. B, Scanning laser densitometric measurements (mean ± SEM) tyrosyl phosphorylation level of the cellular proteins in three independent experiments. The protein bands were arbitrarily divided into two panels to facilitate viewing. The *hatched area* indicates the region in which changes in the steady state tyrosyl phosphorylation level do not differ significantly from the corresponding control. The *dashed lines* represent each corresponding 100% control.

apparent molecular masses of 21, 29, 34, 42, 48, 62, 74, 90, 120, 150, 170, 220, and 240 kDa (Fig. 3). The average densitometric measurements of these cellular proteins in the fluoride-treated cells compared to those in the vehicle-treated cells are shown in Table 1.

Time-dependent effects of fluoride on steady state tyrosyl phosphorylation levels of cellular proteins in human TE85 osteosarcoma cells in vitro

Figure 4, A and B, show the time-course effect of a mitogenic concentration of fluoride (*i.e.* 100 μmol/L) on the steady state tyrosyl phosphorylation level in human TE85 osteosarcoma cells. Figure 4, C and D, represents the average densitometric measurements of the time-dependent changes

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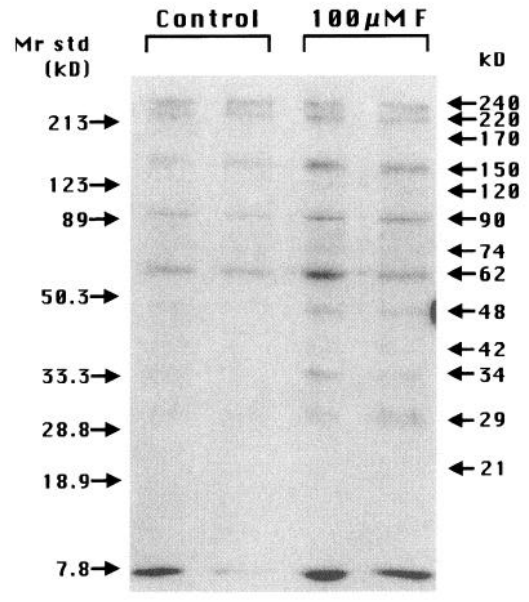

FIG. 3. Effects of a 24-h treatment of normal human mandible-derived bone cells with a mitogenic dose of fluoride on the steady state tyrosyl phosphorylation level of cellular proteins. Human mandible-derived bone cells were treated with 100 μmol/L fluoride for 24 h, and the steady state tyrosyl phosphorylation level was determined by the ECL detection method. The molecular mass standards are shown by the *arrows on the left*; the phosphotyrosyl proteins are indicated by the *arrows on the right*. Relative laser scanning densitometric readings of each individual protein band are shown in Table 1.

TABLE 1. Relative laser scanning densitometric measurements of Fig. 3

Cellular protein (kDa)	Vehicle-treated control (% of vehicle control)[a]	Treated with 100 μmol/L fluoride (% of vehicle control)
240	100 ± 15	122 ± 14
220	100 ± 0	129 ± 12
170	100 ± 6	144 ± 0
150	100 ± 4	214 ± 11
120	100 ± 6	244 ± 17
90	100 ± 6	244 ± 5
74	100 ± 2	240 ± 19
62	100 ± 0	384 ± 216
48	100 ± 28	511 ± 12
42	100 ± 31	325 ± 10
34	100 ± 10	366 ± 40
29	100 ± 13	278 ± 67
21	100 ± 12	241 ± 30

[a] Values are the mean ± SEM of duplicate lanes.

in the steady state tyrosyl phosphorylation levels of the cellular proteins. Treatment with fluoride for 3–24 h significantly increased the steady state tyrosyl phosphorylation levels of at least 13 cellular proteins (*i.e.* 21, 32, 34, 44, 48, 62, 74, 105, 120, 150, 170, 220, and 240 kDa). However, no significant increase in any of these cellular proteins could be detected unless the cells were treated with fluoride for at least 3–6 h. The changes in the steady state tyrosyl phosphorylation levels of the cellular proteins were not due to differences in protein loading, because staining of proteins on the gels with Coomassie blue showed no differences greater than 10% between each sample (data not shown).

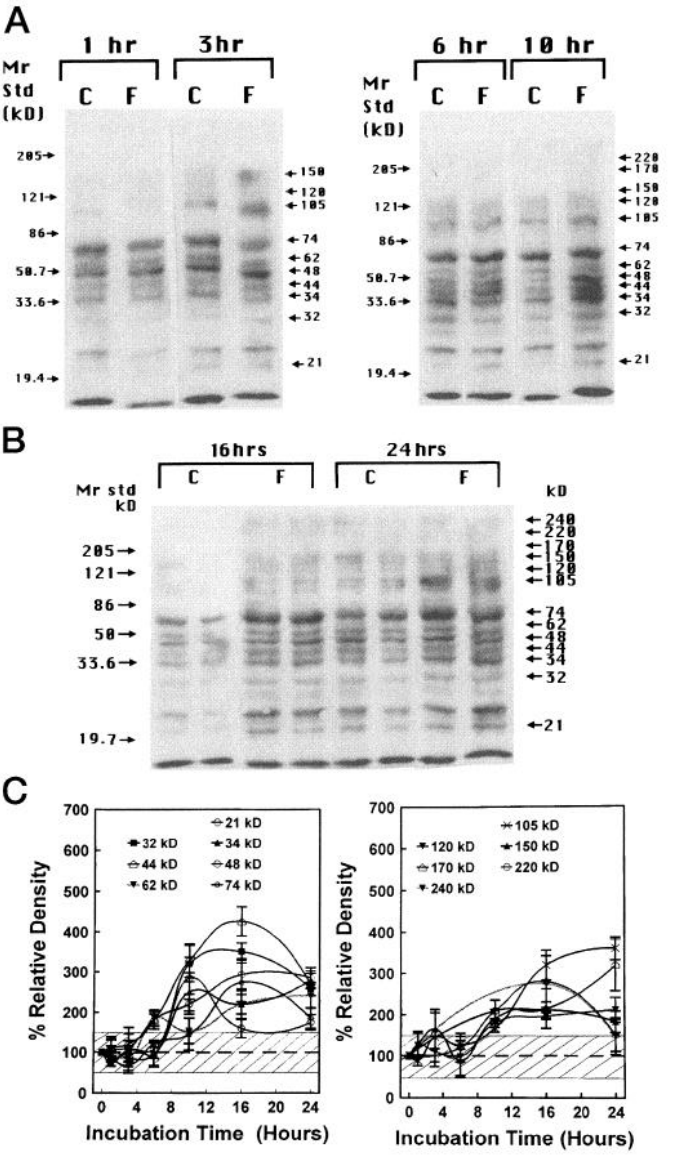

FIG. 4. Time-dependent increases in the steady state tyrosyl phosphorylation levels of cellular proteins in human TE85 osteosarcoma cells by a mitogenic dose of fluoride. Human TE85 osteosarcoma cells were treated with 100 μmol/L fluoride for various length of time (10 min to 24 h). The steady state tyrosyl phosphorylation level was determined using the ECL detection method. A and B, Time-dependent effects of fluoride on the steady state tyrosyl phosphorylation levels of cellular proteins. The results of periods shorter than 1 h are not shown. C, Average scanning laser densitometric measurements (mean ± SEM) of each phosphotyrosyl protein band in duplicate for two independent experiments. The protein bands were arbitrarily divided into two panels to facilitate viewing. The *hatched area* indicates the region in which changes in relative optical density are not considered to be significantly different from the corresponding control value. The *dashed lines* represent each corresponding 100% control.

Time-dependent effects of EGF on steady state tyrosyl phosphorylation level of cellular proteins in human TE85 osteosarcoma cells in vitro

Polypeptide growth factors, such as EGF, are known to stimulate bone cell proliferation and cellular tyrosyl protein phosphorylation. To compare the fluoride-mediated in-

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creases in steady state tyrosyl protein phosphorylation levels and the growth factor-dependent stimulation in cellular tyrosyl phosphorylation, the time-dependent effect of a mitogenic dose (10 ng/mL) of EGF on the steady state tyrosyl phosphorylation levels of cellular proteins was determined in human TE85 osteosarcoma cells (Fig. 5A). Figure 5B represents the relative laser densitometric measurements of the changes in steady state levels of cellular phosphotyrosyl proteins. EGF induced acute (within 10 min) increases in the steady state tyrosyl phosphorylation level of at least 12 cellular proteins with apparent molecular size of 42, 48, 57, 68, 72, 96, 107, 120, 150, 170, 220, and 240 kDa. Although EGF was present throughout the incubation, the increases caused by EGF were transient and rapidly disappeared after 60 min of incubation.

Cell specificity of the fluoride-mediated effects on steady state phosphorylation levels of cellular phosphotyrosyl proteins in vitro

Mitogenic effects of micromolar concentrations of fluoride have been shown to be specific for bone cells, but not cells

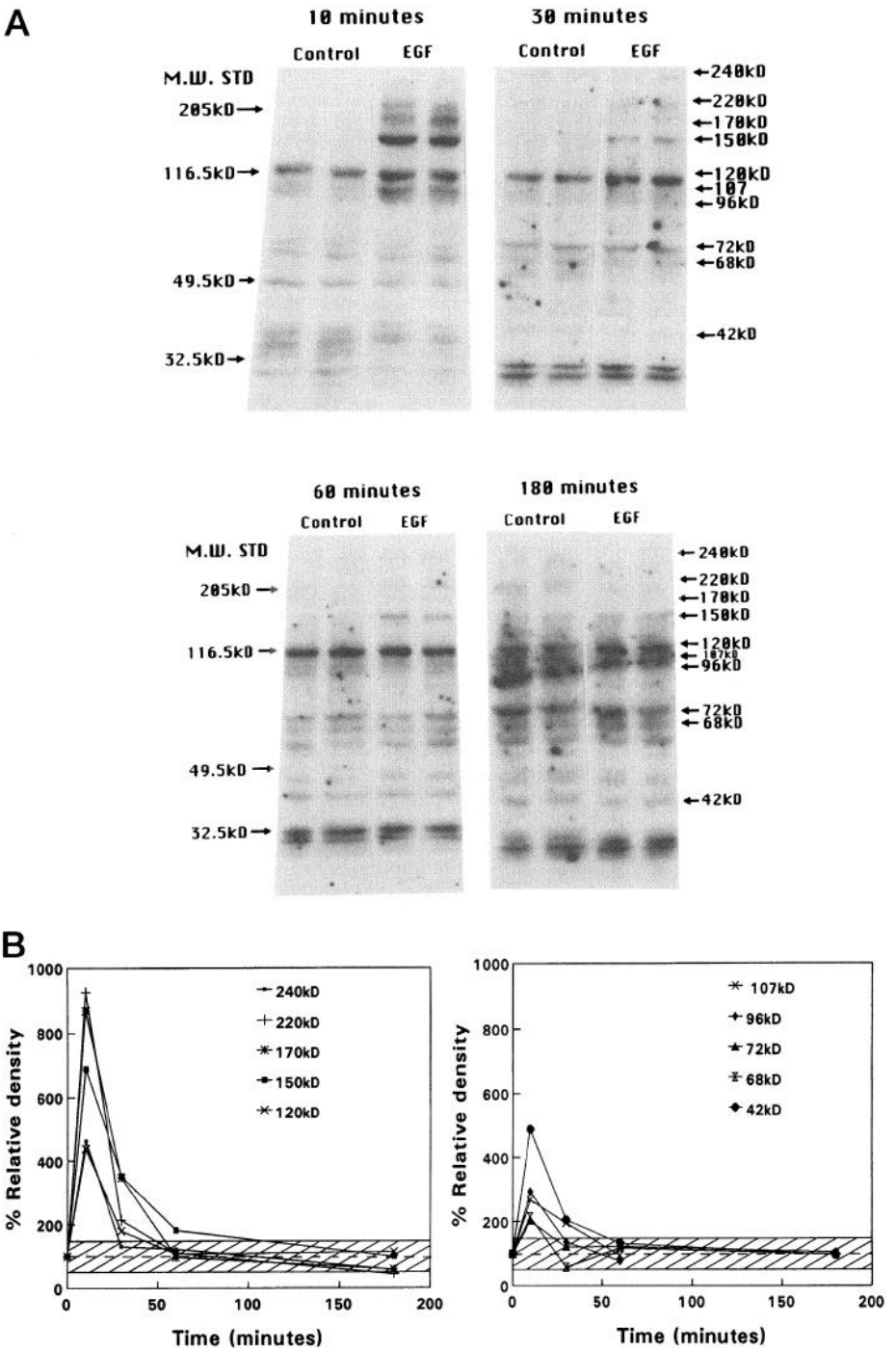

FIG. 5. Time-dependent increases in the steady state tyrosyl phosphorylation level of cellular proteins in human TE85 osteosarcoma cells by a mitogenic dose of EGF. Subconfluent dishes of human TE85 cells were treated with 10 ng/mL EGF for the indicated time. EGF was present in the culture throughout the entire treatment period. The steady state tyrosyl phosphorylation level was determined with the [^{125}I]protein A and autoradiography method. A, Effects of EGF treatment on the steady state tyrosyl phosphorylation levels of cellular proteins. B, Average scanning laser densitometric measurements of cellular proteins whose phosphotyrosyl phosphorylation levels were increased by the EGF treatment. The protein bands were arbitrarily divided into two panels to facilitate viewing. The *shaded area* indicates the region in which changes in the steady state tyrosyl phosphorylation level do not differ significantly from the corresponding control value.

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derived from other tissues (3, 4, 10). Accordingly, we determined and compared the effects of fluoride on the steady state tyrosyl phosphorylation level of cellular proteins in human TE85 osteosarcoma cells and human foreskin fibroblasts. Figure 6 shows that steady state tyrosyl phosphorylation levels of cellular proteins in human TE85 cells were increased by a 24-h treatment with 100 and 200 μmol/L fluoride, which also stimulated the proliferation of these cells in the parallel experiment (data not shown). Conversely, treatment with these same micromolar doses of fluoride did not stimulate the proliferation of human foreskin fibroblasts (data not shown), nor did it induce significant changes in the steady state tyrosyl phosphorylation level of cellular proteins in the skin fibroblasts (Fig. 6). Table 2 shows the relative densitometric measurements of several cellular proteins in these two cell types.

Reversibility of the fluoride effects

We next evaluated whether the effects of fluoride treatment on cellular tyrosyl phosphorylation would be sustained after the removal of fluoride (Fig. 7). Human TE85 osteosarcoma cells were treated with 100 μmol/L fluoride for 21 h, at which time the treatment groups had the fluoride removed by rinsing the culture dishes twice with fresh serum-free DMEM. After that, half of the cells were replaced with serum-free DMEM supplemented with BSA (*i.e.* vehicle control), and the other half was replaced with DMEM containing 100 μmol/L fluoride. The cells were then incubated for an additional 3 h, and the steady state tyrosyl phosphorylation levels of cellular proteins were analyzed. The relative densitometric measurements (mean ± SEM) of the cellular proteins after these treatments are shown in Table 3. Although continuous fluoride treatment for 24 h (*i.e.* 21 h plus another 3 h after medium change) increased the steady state tyrosyl phosphorylation of at least 13 proteins, the fluoride-dependent increases were dissipated after the removal of fluoride for 3 h.

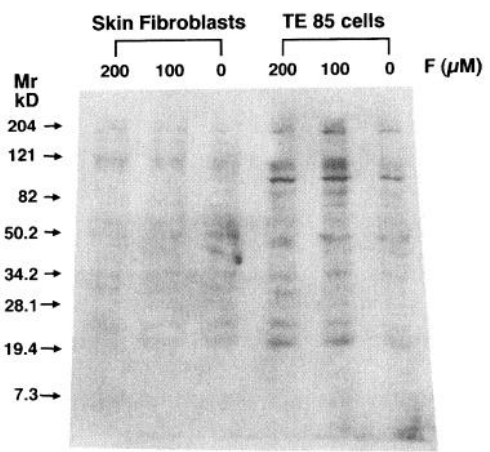

FIG. 6. Comparison of the effect of fluoride on the steady state tyrosyl phosphorylation of cellular proteins in human TE85 osteosarcoma cells with that in human foreskin fibroblasts. Human TE85 cells and human foreskin fibroblasts were treated with 0 (vehicle control), 100, and 200 μmol/L fluoride for 24 h. The steady state tyrosyl phosphorylation level was determined with the ECL detection method. The molecular mass standards are indicated by the *arrows on the left*. Relative laser scanning densitometric readings of each individual protein band are shown in Table 2. This experiment was repeated once. In parallel experiments, fluoride at 25–500 μmol/L did not significantly stimulate [^{3}H]thymidine incorporation into DNA of human foreskin fibroblasts, but 50–200 μmol/L fluoride significantly increased [^{3}H]thymidine incorporation into TE85 cell DNA by 28–49% ($P < 0.05$ for each; data not shown).

Pretreatment of cells with EGF potentiates fluoride's effects

Previous studies indicate that the mitogenic action of fluoride requires the presence of a sufficient amount of tyrosyl kinase-activating growth factors (10, 15, 21). To test whether pretreatment with a mitogenic dose of a growth factor would enhance the early responses to fluoride of steady state tyrosyl phosphorylation levels of cellular proteins, the following experiment was performed. Human mandible-derived bone cells were pretreated with 10 ng/mL EGF (a mitogenic dose) for 5 min. Duplicate plate of cells were then treated with or without 100 μmol/L fluoride for an additional 30 min. Figure 8 shows that pretreatment of normal human mandible-derived cells with EGF for 5 min significantly increased the steady state tyrosyl phosphorylation level of 14 cellular proteins with apparent molecular masses of 21, 29, 32, 34, 42, 48, 62, 74, 90, 120, 150, 170, 220, and 240 kDa. In the presence of 100 μmol/L fluoride during the post-EGF treatment, the steady state tyrosyl phosphorylation level of cellular proteins continued to be elevated. In contrast, the stimulatory effects were dissipated during the 30 min post-EGF treatment without fluoride. The average relative densitometric measurements of these protein bands are shown in Table 4.

Discussion

In this study, we have demonstrated for the first time that treatment with fluoride, at concentrations that stimulated human bone cell proliferation, statistically significantly increased the steady state tyrosyl phosphorylation level of at least 13 cellular proteins in subconfluent monolayer cultures of human bone cells. The fluoride-dependent effects on cellular tyrosyl protein phosphorylation were dose and time dependent. In addition, mitogenic concentrations of fluoride increased steady state tyrosyl protein phosphorylation levels in both transformed human osteosarcoma cells (*i.e.* TE85 cells) and normal human bone cells (*i.e.* mandible-derived bone cells). Thus, the stimulatory effects of fluoride on the steady state level of tyrosyl phosphorylation of cellular proteins are not an aberrant property of osteosarcoma cells, but also seen with normal human bone cells.

Two aspects of the fluoride-dependent effects are noteworthy. First, fluoride at mitogenic concentrations induced increases in the steady state level of tyrosyl phosphorylation of cellular proteins in human bone cells, which are mitogenically responsive to fluoride, but not in human foreskin fibroblasts, which do not respond mitogenically to fluoride (4). These findings suggest that increased tyrosyl phosphorylation levels of cellular proteins may be associated with the cell proliferation process. Accordingly, they provide circumstantial support for the concept that increases in the steady state tyrosyl phosphorylation levels of key cellular proteins in bone cells is a prerequisite for the fluoride-induced stimulation of cell proliferation. Consistent with this hypothesis

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TABLE 2. Relative laser scanning densitometric measurements of Fig. 6

Cellular protein (kDa)	TE85 cells: fluoride (μmol/L)			Skin fibroblasts: fluoride (μmol/L)		
	Control	100	200	Control	100	200
220	100	117	102	100	226	149
170	100	117	102	100	362	238
120	100	102	98	100	485	270
105	100	71	47	100	246	179
90	100	84	40	100	173	151
74	100	102	59	100	139	112
48	100	125	112	100	171	161
34	100	108	72	100	152	112
32	100	108	83	100	256	204
24	100	84	88	100	566	597
21	100	117	109	100	265	307

Results are shown as a percentage of corresponding vehicle-treated control value.

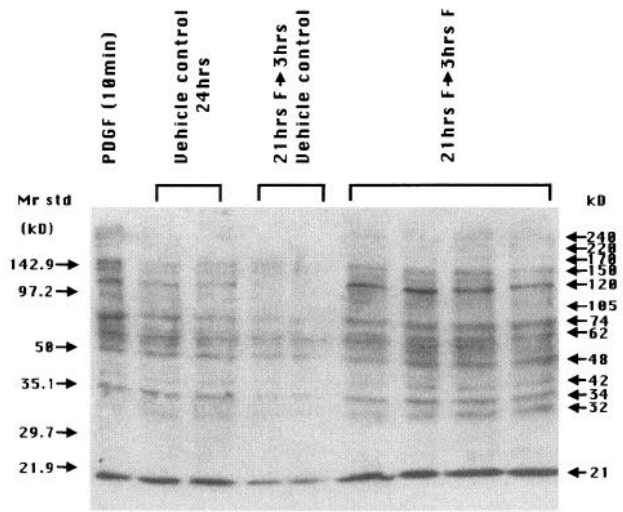

FIG. 7. Reversibility of fluoride-mediated increases in the steady state tyrosyl phosphorylation level of cellular proteins. Human TE85 osteosarcoma cells were treated with or without 100 μmol/L fluoride for 21 h, at which time the fluoride-treated plates were rinsed twice with fresh DMEM to remove fluoride. Half of the rinsed cell plates were replaced with fresh DMEM, whereas the other half was replaced with fresh DMEM supplemented with 100 μmol/L fluoride. All cells were further incubated in their respective medium for an additional 3 h (*i.e.* total incubation = 24 h), cell extracts were immediately prepared, and the steady state tyrosyl phosphorylation levels of cellular proteins were determined with the ECL detection assay. The same cells treated with 10 ng/mL platelet-derived growth factor for 10 min were included in this experiment as a positive control. The molecular mass standards are indicated by the *arrows on the left*, and those cellular proteins whose tyrosyl phosphorylation level was increased were shown by the *arrows on the right*. Relative laser scanning densitometric readings of each individual protein band are shown in Table 3. This experiment was repeated twice with same results.

is the recent finding that genistein, a tyrosyl kinase inhibitor, inhibited the fluoride-induced cell proliferation (22). Second, the effects of fluoride were readily reversible, as most fluoride-induced increases in the steady state tyrosyl phosphorylation level of cellular proteins disappeared after the removal of fluoride. This suggests that fluoride must be present continuously to produce optimal effects on the steady state tyrosyl phosphorylation level and perhaps also on cell proliferation. These observations could be clinically relevant, because they suggest that to produce an optimal effect in the treatment of osteoporosis, the serum fluoride concentration should be maintained continuously at the therapeutic level.

Increased steady state tyrosyl phosphorylation levels could be mediated by activation of tyrosyl kinases, inhibition of PTPPs, or both. In this regard, the effects of a direct activation of tyrosyl kinases, *e.g.* by a polypeptide growth factor, on the tyrosyl phosphorylation level are expected to be acute and transient (due to the counterregulatory actions of PTPPs) (23, 24). In support of this concept, the present study shows that treatment of human TE85 osteosarcoma cells with a mitogenic dose of EGF led to a rapid, but transient, increase in the steady state tyrosyl phosphorylation level of cellular proteins. On the other hand, the effects of inhibition of PTPPs would probably be slow in onset (depending on the basal activity of tyrosyl kinases), but sustained. In agreement with this speculation, our recent preliminary studies showed that the stimulatory effects of orthovanadate, a well known PTPP inhibitor, on steady state tyrosyl phosphorylation level of cellular proteins in human TE85 osteosarcoma cells was indeed slow in onset (it took 3–6 h to increase) and was sustained for up to 24 h of treatment (unpublished observations). In this study, the stimulatory effects of fluoride on cellular steady state tyrosyl phosphorylation levels, like those of orthovanadate, were shown to be slow in onset (*i.e.* 3–6 h) and sustained for at least 24 h. This observed time course is more compatible with the premise that the action of fluoride on cellular tyrosyl phosphorylation is mediated by an inhibition of one or more fluoride-sensitive PTPPs than through a direct activation of tyrosyl kinases.

It should also be noted that the effects of fluoride on the steady state tyrosyl phosphorylation levels of cellular proteins in human bone cells were greatly enhanced by brief pretreatment with a tyrosyl kinase-activating growth factor, such as EGF, suggesting an interaction between EGF and fluoride. Although the stimulatory effects of the EGF pretreatment rapidly disappeared without the subsequent fluoride treatment, the stimulatory effects of EGF pretreatment were sustained as long as the cells were exposed to fluoride. These findings are consistent with our previous findings that the mitogenic effects of fluoride require and interact with a tyrosyl kinase-activating growth factor (15, 21). Moreover, that fluoride has no effect on protein thiophosphorylation (10) is consistent with the interpretation that fluoride has no direct stimulatory effect on protein kinases. Together, these findings lend further circumstantial support for our hypoth-

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TABLE 3. Relative laser scanning densitometric measurements of Fig. 7

Cellular protein (kDa)	Vehicle controls (% of control)[a]	Discontinuous fluoride treatment (% of control)[a]	Continuous fluoride treatment (% of control)[b]
240	100 ± 2	32 ± 32	333 ± 28
220	100 ± 8	36 ± 12	171 ± 12
170	100 ± 29	46 ± 27	196 ± 23
150	100 ± 5	75 ± 9	171 ± 3
120	100 ± 2	42 ± 5	239 ± 8
105	100 ± 3	39 ± 1	168 ± 11
74	100 ± 18	37 ± 1	177 ± 13
62	100 ± 17	32 ± 1	157 ± 9
48	100 ± 4	44 ± 2	153 ± 6
42	100 ± 7	21 ± 2	193 ± 6
34	100 ± 3	26 ± 4	148 ± 10
32	100 ± 4	34 ± 8	244 ± 13
21	100 ± 7	35 ± 5	267 ± 24

[a] Mean ± SEM of duplicate lanes. TE85 osteosarcoma cells were treated with 100 μmol/L fluoride for 21 h, at which time the cells were rinsed twice with fresh DMEM to remove fluoride. The fluoride treatment was discontinued by incubating the rinsed cells in fresh DMEM for an additional 3 h.

[b] Mean ± SEM of four replicate lanes. TE85 cells were treated with 100 μmol/L fluoride for 21 h. The fluoride-treated cells were rinsed twice with fresh DMEM, followed by further incubation in a fresh medium containing 100 μmol/L fluoride for an additional 3 h.

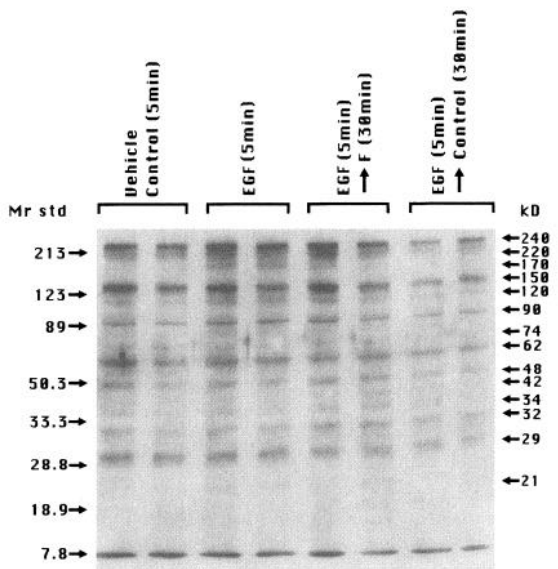

FIG. 8. Effect of EGF pretreatment on the ability of fluoride to acutely increase the steady state tyrosyl phosphorylation level of cellular proteins. Human mandible-derived bone cells were pretreated with 10 ng/mL EGF for 5 min, after which the medium was changed, and half of the cells were incubated with medium containing 100 μmol/L fluoride for 30 min. The other half were incubated with medium containing no fluoride for 30 min. The steady state tyrosyl phosphorylation level of cellular proteins was determined with the ECL detection assay. The molecular mass standards are indicated by the *arrows on the left*, and those cellular proteins whose tyrosyl phosphorylation level was increased are shown by the *arrows on the right*. The relative laser scanning densitometric readings of each individual protein band are shown in Table 4. This experiment was repeated once with identical results.

esis that the mitogenic action of fluoride is mediated through an inhibition of phosphotyrosyl protein dephosphorylation and not through a direct activation of tyrosyl kinases.

In conflict with our hypothesis, a recent report suggested that the mitogenic action of fluoride in rat UMR106 bone cells is mediated by a direct activation of tyrosyl kinase activities (22). This conclusion was based on three key findings: 1) genistein blocked the mitogenic action of fluoride; 2) treatment with fluoride for 18 h increased the *in vitro* tyrosyl kinase-specific activity using poly(Glu,Tyr) as the substrate; and 3) fluoride had no *in vitro* inhibitory effect on the UMR106 cell membrane-mediated dephosphorylation of [^{32}P]tyrosyl-phosphorylated myelin basic protein. On the basis of these findings, it was concluded that fluoride's action involves a direct activation of tyrosyl kinase activity. We believe that this conclusion may be an overinterpretation of their data for three reasons. First, it has been shown that the mitogenic action of fluoride on bone cells requires a tyrosyl kinase-activating growth factor (15, 21). Thus, an alternative explanation is that genistein indirectly abolished the mitogenic effects of fluoride by blocking the action of the growth factor that is required to raise the basal tyrosyl phosphorylation rate for fluoride to effectively increase the steady state tyrosyl phosphorylation level of cellular proteins by inhibition of PTPP. Consistent with this interpretation, our recent preliminary studies showed that tyrphostin, another tyrosyl kinase inhibitor, abolished the mitogenic activity of phenylarsine oxide, a known PTPP inhibitor (25). Second, it is puzzling why an 18-h treatment with fluoride was required for an activation of tyrosyl kinase activity if the primary action of fluoride was to directly activate tyrosyl kinase activities. In this regard, treatment of bone cells with growth factors that directly activate tyrosyl kinases induced immediate, but transient, activation of tyrosyl kinase activity. Thus, if the main effect of fluoride is mediated by a direct activation of tyrosyl kinases, one would expect the effect to have occurred much earlier (*i.e.* within minutes of fluoride treatment) than 18 h. The effects would also be expected to be short lived (due to the countering actions of PTPP) and not sustained for 18 h. [Many tyrosyl kinases are shown to be activated by autophosphorylation (24, 26). Thus, fluoride treatment could indirectly activate these kinases by preventing the dephosphorylation of their autophosphorylated tyrosine residues. Accordingly, even if there were an increase in tyrosyl kinase activity, this could be a secondary, rather than a primary, action of fluoride on the tyrosyl kinases.] Third, [^{32}P]tyrosyl-phosphorylated myelin basic protein is a poor substrate for many PTPP. Thus, it is foreseeable that the

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TABLE 4. Relative laser scanning densitometric measurements of Fig. 8

Cellular proteins (kDa)	Vehicle control (% of control)	EGF alone (% of control)	EGF, then fluoride (% of control)	EGF, then control (% of control)
240	100 ± 15	145 ± 15	129 ± 13	37 ± 6
220	100 ± 12	154 ± 2	152 ± 18	70 ± 1
170	100 ± 1	331 ± 6	288 ± 32	64 ± 20
150	100 ± 15	113 ± 8	125 ± 22	47 ± 15
120	100 ± 35	216 ± 2	197 ± 47	45 ± 28
90	100 ± 25	150 ± 4	124 ± 28	70 ± 10
74	100 ± 17	154 ± 4	162 ± 30	86 ± 33
62	100 ± 12	115 ± 15	106 ± 5	71 ± 9
48	100 ± 33	92 ± 9	106 ± 18	65 ± 3
42	100 ± 28	105 ± 15	140 ± 36	82 ± 12
34	100 ± 21	126 ± 23	158 ± 19	95 ± 14
32	100 ± 14	122 ± 11	155 ± 4	120 ± 2
29	100 ± 12	104 ± 6	109 ± 3	68 ± 19
21	100 ± 20	172 ± 42	189 ± 33	85 ± 16

Values are the mean ± SEM of duplicate lanes.

lack of inhibition by fluoride of PTPP activities in UMR106 cell extracts may be a result of the use of an inappropriate substrate. Nevertheless, although we believe the possibility that fluoride acts through a direct activation of tyrosyl kinases is remote, we cannot entirely rule out this possibility. Additional work is required to resolve this apparent discrepancy.

The identity of the cellular proteins whose steady state tyrosyl phosphorylation level was increased by fluoride is not known at this time. An effort is underway in our laboratory to determine their identities and potential functions. Regardless of their identities, these findings represent the first evidence that fluoride, at concentrations that stimulated human bone cell proliferation *in vitro*, significantly increased the steady state tyrosyl phosphorylation level of at least 13 cellular proteins in human bone cells *in vitro*, and that the time course of the fluoride effect is consistent with the interpretation that the action of fluoride is mediated by the inhibition of dephosphorylation rather than a direct activation of phosphorylation. These findings together with our previous observations (10) are entirely compatible with our overall model that fluoride stimulated cell proliferation by inhibiting the activity of one or more osteoblastic fluoride-sensitive PTPP.

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